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MICRO-TECHNIQUE

Suggestions for Methods and Apparatus

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I

SYSTEMATICALLY EXAMINING LARGE SERIES OF MICROSCOPICAL OBJECTS

There are various methods of recording the position and character of each member of a large series of objects mounted on a microscope slide. One of the commonest methods involves the use of a recording, mechanical stage. Each object on the slide receives a record-number consisting of two separate readings from scales engraved on the mechanical stage. The following method, however, is successful without a mechanical stage or finder of any sort, and is characterized by simplicity and expedition. It may be called the method of charting.

The method consists in making a camera lucida drawing or chart, at low magnification, of all the objects of which it is desired to make record. The chart is diagrammatic; each object is represented on the chart by a simple, characteristic diagram, and the diagrams are then numbered in series. The sheet that carries the chart may also carry a series of printed numbers with corresponding spaces for records. (See Figure 1.) Where the objects belong to a few great groups, such as land-inhabiting, fresh-water, and marine, the printing of the blank sheets in correspondingly assorted colors is an advantage.

The chart is made by using a camera lucida and an objective of about five-inch focus.[1] In order to reduce the magnification, the objective may be screwed into the end of the draw-tube of the microscope barrel. A low power eye-piece is used with the objective, so

[1] A very strongly magnifying spectacle lens will serve the purpose.

that all the objects on the slide can be seen at one time. A chart having a magnification of five diameters is of convenient size. The suitable illumination is secured by using a concave mirror without sub-stage condenser. The light may be direct, in which case the objects are seen as dark bodies on a light background, or a dark-ground effect can be produced by inserting between the concave mirror and the objects a small opaque disc. A suitable disc may be made by stripping the barbules from a dark-colored six-inch wing or tail feather so

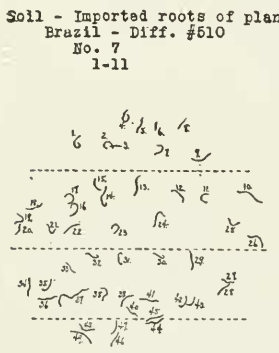

Fig. 1. Record chart used in tabulating large numbers of microscopic objects arranged on a series of slides. As printed the chart was 5x8 inches, and carried only the two columns of figures 1 to 50 inclusive. At the left is seen the camera lucida drawing, or chart, recording the form, size, and relative position of forty-nine microscopic objects, —in this particular case, nemas. Immediately above the chart are seen the data relating to the particular slide charted, which was No. 7 in a series of eleven slides (1-11), and which carried a collection of forty-nine nemas gathered from soil attached to the roots of plants imported from Brazil. Names and other notes with regard to the nemas were typewritten opposite the appropriate numbers. Nos. 2, 7, 12, 13, 14, 23, 48, and 49 were encircled to indicate that these specimens were of especial interest. One-half size.

as to leave only a small fan-shaped tip at the end, from one-half to three-fourths of an inch across. With scissors, this is trimmed so as to have a somewhat rounded contour. While the right hand is engaged in making the chart, the left hand can flirt this little disc in and out between the objects and the concave mirror and so produce a

is sawed from a sheet of German silver about one two-hundredth of an inch thick. The edges of the central aperture are beveled so that the mixture frozen on it becomes dove-tailed to the plate. In a similar way, the small, washer-shaped piece of German silver fastened to the top of the dome, as shown in Fig. 3, r, is also beveled.

The German silver wheel is soldered throughout to a round sheet of exceedingly thin brass or German silver. Then into six marginal perforations in the German silver wheel, brass pins are soldered, giving to the whole affair the appearance of a six-legged table. The heads of the pins are filed off so as to give clearance for the microtome knife. The pins serve to fasten the plate to a perforated cork, being thrust into the cork as shown in the illustration. The rim of the dome of thin sheet metal is somewhat similarly stiffened by soldering to it a ring of German silver which is perforated and supplied with six brass pins in the manner just described.

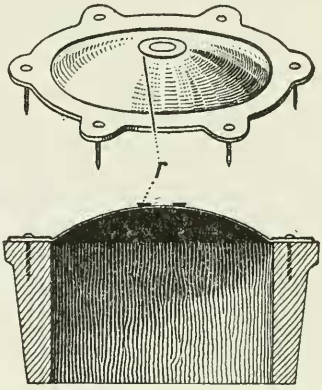

Fig. 3. Perspective view and longitudinal section of a freezing-microtome object-holder mounted on a cork cylinder. The holder is made of metal only about 2/1000 of an inch thick. The edges of the ring (r) are beveled so that the imbedding mixture when frozen is dovetailed to the holder.

Though the dome-form is somewhat more difficult to construct than the flat, it is more efficient for three reasons: It is more rigid, it gives a better clearance for the microtome knife, and it contains less material.

In the case of small and moderate sized objects of which only a few sections are required, the method is extraordinarily expeditious. Objects of such a size that they can be imbedded in a few drops of the freezing mixture placed on the control part of either of these metal supports can be frozen in a few seconds by applying an ordinary ether spray to the under side of one of these thin metal supports. The exceeding rapidity of the congelation gives rise to a consistency favorable to section cutting.

III

TO OBTAIN AN END VIEW OF A NEMA, ROTIFER, OR OTHER SIMILAR SMALL OBJECT

Suppose the object is a nema of which an end view of the head is required: decapitate the nema behind the pharynx with the aid of an eye knife, or similar very small tool, having a very slender, thin blade. The smallest and most slender-bladed knife used by oculists in operations on the eye is a very suitable tool, and it must have the degree of sharpness characteristic of surgical instruments in good order. Bring the nema by appropriate methods into glycerine; the decapitation should be done in a drop of glycerine placed on the surface of a transparent piece of celluloid. Push the nema to the bottom of the glycerine and against the celluloid; decapitate by pressing the edge of the knife against the nema as the latter rests on the celluloid. The celluloid is sufficiently soft so that the edge of the knife will not be dulled. If the knife is sharp, the cut will be clean, and the object satisfactory. If the knife is dull, the nema will be more or less crushed at the point of section and the preparation may prove unsatisfactory.

Mount the head in melted glycerine jelly, using sufficient jelly so that the object may stand on end after being covered in. Place the mount on the stage of a microscope, bring the object into focus, and with a dissecting needle gently shove the cover-glass slightly back and forth until the object is seen to be on end. Allow to remain on the stage of the microscope until the jelly sets, watching from time to time to see that the object maintains the desired position.

According to my experience, this is a better method of obtaining end-on and sectional views of the heads of free-living nemas and other similar small organisms than that of sectioning and imbedding. The trouble with the method of sections is that the microtome knife very seldom cuts the object to advantage. It is quite likely to cut in the wrong place. If the ends of the setae or the surfaces of the lips are removed in the first cut, it is a very troublesome matter to obtain a good view or good sketch of the structures. Even if some of the parts should not be lost or offer difficulty in mounting, there are so many chances that the microtome blade will cut through at a disadvantageous place that, as a rule, a very considerable number of nemas will have to be sectioned before a good preparation is secured.

The method of sections has the further disadvantage that the following of such small objects through the various dehydrating and

staining fluids, and the final orientation of them, is a tedious and difficult matter. Moreover in the case of nemas, there is considerable difficulty in properly imbedding the object. The cuticle of nemas is so impenetrable that unless special precautions are taken, the paraffine will not thoroughly penetrate the tissues, and the results will be unsatisfactory.

End views may be obtained by mounting the nemas in a microscopic well made from a thin section of thermometer tubing. The tubing should be like that used in the most delicate medical thermometers, that is to say, with the smallest aperture procurable. This tubing may be bought under the name thermometer, or barometer tubing. It is well to have on hand ground sections of varying thickness, from one-quarter of a millimeter thick to one millimeter or more. The discs are cemented to a glass microscope slide at the time of using by means of smoking hot wax or other suitable cement. Before cementing the disc to the slide, fill the capillary aperture in the disc with mounting fluid. This may be easily done by placing on the slide a very tiny drop of the mounting fluid, and laying the disc onto the small drop. The mounting fluid will enter the aperture by capillarity. If it be desired to look at the head end of a nema, it is placed in the microscopic well, tail down. If the nema is too long for the well, it may be cut to fit it. The point is, to see that the object has about the same length as the depth of the well, so that the end portion of the object it is desired to view will come close to the under side of the cover-glass when this latter is placed on the top of the well, or rather on the disc of the glass containing the well. In placing the nema in the well, a suitable tool is a small, curved hair cemented to the end of a dissecting needle. Human eye-brow hairs are suitable for this purpose. Using this method, the specimen can be examined in clove oil, cedar oil, or any mixture of these or any other similar thin mounting fluid. Cedar oil, having the same refractive index as the glass composing the well, has advantages in connection with illumination. The illumination in aqueous media is less satisfactory.

When the glass discs are not in use, it is best to keep them in absolute alcohol in a glass-stoppered bottle. They should not be allowed to become dry with mounting fluid in the capillary orifice, otherwise they will be very troublesome to clean out.

IV

DESTAINING OF NEMAS OR OTHER SMALL OBJECTS IN THE DIFFERENTIATOR

In handling a mass of small organisms by the differentiator method, there is sometimes considerable difficulty in securing satisfactory destaining. There is little difficulty in getting a mass of organisms thoroughly impregnated with the stain, no matter how varied they may be in species and in size; it is simply a matter of time. The trouble comes in destaining. If the destaining process is carried on until the largest of the objects, or the most impenetrable ones, are sufficiently destained, it will generally happen that smaller specimens, or those more easily penetrated, are deprived of too much of their color. It is therefore a matter requiring considerable experience and judgment to successfully destain such a miscellaneous collection. The difficulty is considerably increased by the fact that when enclosed in the differentiator tube, the specimens are not very easy to examine critically by any ordinary method. If the differentiator be held toward a strong light, the organisms may be examined by the aid of an ordinary pocket lens, but not very critically. The most satisfactory piece of apparatus for this work is what is sometimes known as the chemical microscope, in which the objective is below the stage and the light that passes through it from above is reflected by a prism placed below so as to pass obliquely upward through a barrel carrying an eye-piece. If the differentiator tube containing the destained nemas is laid on a glass stage over the objective of such an inverted microscope, and a little water, or still better, cedar oil, be placed between the differentiator tube and the glass stage, it will be found that the nemas or other objects will sink to the bottom of the fluid in the differentiator tube so as to come as near as possible to the objective of the microscope. If the glass stage is thin, there is no difficulty in using a one-half to two-thirds inch objective. In this way, the nemas may be examined more critically with regard to the extent of the destaining.

If it is desired to use a lens of higher power, it is sometimes possible to do so by resorting to another method. Place a cover-glass on a horizontal surface, and on the cover-glass a good-sized drop of cedar oil. Lay the differentiator tube into this drop of cedar oil in such a way that the nemas come opposite the cover-glass. It will now be

dark-ground effect as desired. To do this the feather "disc" must be materially smaller than the mirror.

The charts are nothing more than rude camera lucida drawings of the objects, and with practice can be made with great rapidity. A lot of fifty nemas mounted under a three-quarter inch round cover-glass can be drawn in two to three minutes with sufficient accuracy to make a very useful chart. (See Figure 1.) Each nema-diagram on the chart has four very distinct properties, (1) Position, (2) Form, (3) Size, (4) Orientation. For the most satisfactory work, it is desirable that a certain optimum number of objects exist on the slide. This optimum is determined by the number of them that will appear in a single field of the lens afterward used in searching. Suppose a sixteen millimeter objective is used as a searching objective, and a four millimeter for the examination; then the optimum number of objects under the cover-glass is that number which brings into each field of the sixteen millimeter objective one to three objects.

After the chart is made, the short, crooked lines, representing the nemas, say, are numbered in transversely arranged groups. Each transverse group of the series constitutes a band of nemas running across the mount and having such width as comes fairly well within the scope of a single field of the 16 mm. objective. These imaginary bands are illustrated in Figure 1. It will be seen that there are four such bands. The nemas are numbered more or less consecutively. Proceeding in this manner, on reaching the end of the first band, one numbers the second band, also more or less consecutively, and so on to the end.

In recording, begin with No. 1, placing it in the field of the 16 mm. objective. It is recognized by its size, form and orientation. Having recorded No. 1 and examined it with the 4 mm. objective, a glance at the chart will indicate at what distance, and in what direction, No. 2 lies from No. 1. Revolving to the 16 mm. objective and looking through the microscope at Nema No. 1, the slide is moved in the indicated direction until No. 2 is found and recognized. After recording No. 2, No. 3 is found in the same way, and so throughout. The novice will be surprised to find how easy it is, with a little practice, to follow the series through without error.

The drawings should be so made and numbered that the chart and the objects as seen under the microscope will resemble each other.

If no care be taken in this respect, the chart may be found to be "left-handed." Securing a "right-handed" chart is merely a matter of properly arranging the paper at the time the chart is drawn. Diagrams should be so made with reference to the printed matter that when it is right side up, the objects as viewed through the microscope will have the same orientations as the diagrams.

This completes the description of this method, except to explain that in the example illustrated, the numbers encircled are so marked in order to indicate that those particular specimens present noteworthy features.

The method may be elaborated in a variety of ways for the recording of nemas, rotifers, protozoa, desmids and a vast array of other microscopic objects. If the charts are of card-system size, say 5x8″, they lend themselves to all sorts of convenient methods of filing. By using thin paper, carbon copies can be made at the original draft.

The charts can be made and used by a grade of assistant that might hardly be intrusted with the use of a recording mechanical stage, and who may lack training in the accurate reading of scales and the recording of numbers. Floating of the objects, of course, disarranges them. Newly made slides are sometimes subject to this disadvantage. The difficulty is avoided by keeping the slides always in a horizontal position.

II

OBJECT SUPPORT FOR A FREEZING MICROTOME

In this freezing microtome attachment, the object is to reduce the metal parts to a minimum and to concentrate the effects of the freezing mixture as much as possible upon the object to be frozen. To this end the object is placed on a thin metal plate, only about one to three thousandths of an inch thick, to which the necessary rigidity is imparted either by soldering it to a radiating framework in the form of a flat wheel sawed from somewhat thicker metal, or, preferably, by giving to the metal the form of a dome. These metals supports are illustrated in Fig. 2 and Fig. 3 in which they are shown full size. A six-spoked wheel, having a hub-hole one-eighth of an inch across,

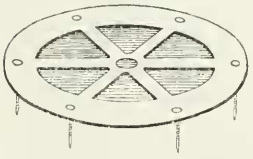

Fig. 2

found that the cover-glass will adhere to the differentiator by capillarity, so long as the differentiator is held in a horizontal position. If the chemical microscope stage has a large aperture, it will be possible to lay the differentiator across the stage, cover-glass downward. In this way, if the differentiator tubing is thin, it will be possible to use even quarter-inch objectives of long focus.

Where considerable work is done with differentiators, a chemical microscope used in this way is a valuable accessory.

V

COMPRESSORIUM FOR CHROMOSOMES

When chromosomes or other similar minute bodies are so massed together that one lies behind another and is thus liable to be missed in counting, the compressorium described below may prove useful in overcoming the difficulty, which none of the ordinary compressoria will do.

When such a mass of chromosomes is flattened out by pressure, the individual chromosomes behave somewhat as would the seeds of a pulpy fruit under similar circumstances. They appear to be of a different consistency from the material in which they lie, and behave under pressure as if harder and more compact than the surrounding matter. Under moderate pressure they do not show much tendency to break in pieces, but rather to accommodate themselves to the narrower quarters by rearranging themselves more nearly in one plane. So far as enumeration of the chromosomes is concerned, this new arrangement has two advantages: 1st, they may all be more readily brought into a single view, that is, all brought into focus at one time; 2nd, in the flattening-out process, they slip one over another somewhat, and recede from each other—for instance, as the seeds inside a grape will do, when similarly pressed.

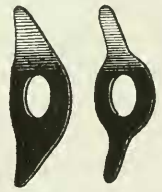

Fig. 4. Two curved, perforated, steel springs made from thin, safety-razor blades, as described in the text. These two forms, while of the same length, nearly one inch, are of different degrees of springiness; that at the left being the weaker.

The compressorium I have devised to secure this effect is constructed as follows: Take a safety-razor blade—one of the thinnest kind, having perforations an eighth of an inch in diameter—and

soften it by heating it to a red heat. With shears, cut a somewhat diamond-shaped piece from the softened blade, so that the "diamond" is about three to four times as long as wide, and has one of the round apertures in its center; bend this elongated "diamond" into a symmetrical bow whose depth is one-eighth of an inch or more. See Fig. 4. Heat the bow in a flame to a cherry-red and plunge it into cold oil or water to harden it. This will result in a springy piece of metal that can be utilized to exert pressure on a small cover-glass under which are mounted cells containing the chromosomes it is desired to scatter. The length of the piece of springy steel may conveniently be made to be about one inch, so that it will just reach across an ordinary three-by-one glass microscope slide. Bind the slide in a piece of thin metal having a three-quarter inch perforation at the back—that is to say, so bend a piece of thin sheet metal that an ordinary slide will slip into it through grooves along the two sides of the folded piece of metal. See Fig. 5. This metal should simply pass around the edges of the slide and lap over about a sixteenth of an inch at each edge leaving one face of the slide uncovered. The grooves should be a little wider than the thickness of the slide—at least enough wider so as easily to admit the thin perforated metal spring. Place the cells, the chromosomes of which are to be studied, on the slide opposite the middle of the three-quarter inch aperture. Use very little mounting medium; cover the cellular tissue to be treated with a small round cover-glass. Tuck the ends of the bowed piece of springy perforated steel under the edges of the metal slide-case or holder, holding the spring against the small cover-slip in such a way that the cells to be compressed lie opposite the center of the small perforation. Press and lock the spring in the same way as in the case of the springs at the back of an ordinary photographic printing frame. The cells will now be under pressure at or near the center of the perforation in the steel spring. The entire contrivance will differ but very

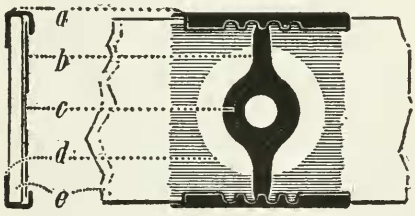

Fig. 5. Portion of a 3x1 inch glass microscope slide enwrapped with thin metal as described in the text. *a*, thin metal wrapper; *b*, one of the springs shown in Fig. 4, placed in position on the slide so as to press the small round cover-glass, *c*, against the slide, *e; d*, aperture in the back of the metal wrapper, *a*. The ends of the spring, *b*, enter through the notches on the edges of the wrapper, *a*, so that in being applied the spring does not need to be rotated more than a few degrees.

little in form and size from an ordinary microscope slide and can be placed on any microscope stage in the same way as a slide. The piece of springy steel is so thin that it in no way prevents the use of a high-power immersion objective. Needless to say, it is for this reason that it is made from such thin metal. The spring may be manipulated with the aid of matches or wooden toothpicks.

Ordinary slides and cover-glasses are almost never perfectly flat. Better results will be obtained by this method if the slide has its convex surface up and the cover has its convex surface down, so that the cellular tissues to be treated lie between two very slightly convex surfaces. It will be found that in this way very compact groups of chromosomes and other similar objects can sometimes be scattered so as to be counted, when otherwise they could not be counted.

There seems to be comparatively little danger of exerting too much pressure. The beginner's tendency at first is to exert, if anything, too little pressure. The greatest difficulty arises from sliding the glasses on each other, since much of this ruins the preparation. To overcome this difficulty, a series of three or four notches, close together, may be filed in the edges of the metal holder before it is folded about the slide,—or rather about the metal core on which it is bent, or formed, and which naturally has a little greater width and thickness than the slide. If now the bowed spring has a length a little less than the distance between the bottoms of the notches in the edges of the slide-holder, it will be found when it is pressed down that the pointed ends can be tucked through the notches and under the edges of the holder without materially sliding or rotating the spring. The accompanying illustrations will assist in understanding this simple and effective device.

The particular cells to be compressed are prepared and searched out in the usual way, then dissected out together with as little of the surrounding tissue as possible, an operation performed with the aid of an ordinary dissecting microscope. It may be advisable to look at the group of chromosomes from both sides. To do this, the metal holder, instead of having a three-quarter inch perforation, should have a much smaller perforation, say about one-eighth of an inch. Instead of using a three-by-one glass slide, cement to the inside of the metal holder a thin cover-glass several sizes larger than that to be

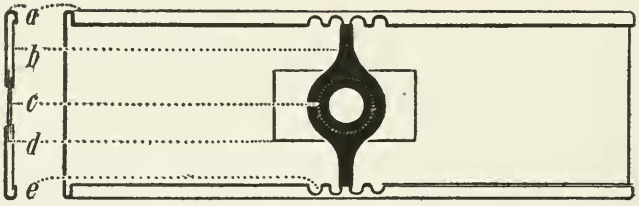

Fig. 6. A metal holder for clamping a microscopic object between two thin cover-glasses. *a*, metal holder; *b*, steel spring as illustrated in Figs. 4 and 5; *c*, small, round cover-glass; *d*, rectangular cover-glass underneath the round cover-glass; *e*, notches in the metal holder for the reception of the spring. This holder enables the microscopist to look at the object with an immersion lens from either direction.

placed over the object. As the metal holder, in order to be stiff enough, has to be several times thicker than the bowed spring, it may be advisable to bevel the edge of the round aperture in the holder, so that it will interfere as little as possible with the use of an immersion objective. On a slide constructed in this manner, the object is held between two cover-glasses, and hence may be viewed from either side with equal ease. Such a slide furthermore permits the use of an immersion lens as a condenser, a proceeding that has advantages.